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(54) Title: PHARMACEUTICAL COMPOSITION FOR THE PREVENTION OF NEURONAL CELL DEATH

(57) Abstract

Pharmaceutical composition suitable for the prevention of neuronal cell death comprising 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline and a diuretic agent and methods of protection against said cell death are disclosed.

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Pharmaceutical Composition for the Prevention of Neuronal Cell Death

The present invention relates to pharmaceutical preparations having the ability to protect against neuronal cell death associated with neuronal focal or global ischemia, oedema, or traumatic events. The invention also relates to a method of protection against neuronal cell death associated with neuronal focal or global ischemia, oedema, or traumatic events. Such conditions occur in subjects suffering from stroke, cardiac arrest, suffocation or traumatic injury of the brain or spinal cord.

It is known that NBQX (2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline) is useful in the treatment of indications caused by hyperactivity of the exitatory neurotransmitters, US Pat. No. 4,889,855. NBQX has proved to be effective in animal models of cerebral global ischemia (Sheardown et al., Science, **247**:571, 1990; Buchan et al. Neurosci. Lett., **132**: 255, 1991) and focal ischemia (Buchan et al. NeuroReport, **2**:473, 1991). The neuroprotecting effect is due to blockade of especially the AMPA receptor, which is a subtype of the glutamate receptor. Even 8 hours after the ischemic insult treatment with NBQX has been shown to be reduce the injury in the hippocampus of rat brain (Pulsinelli et al., Neurology **42**: Suppl. 3:532S, 1992 (Abstract)). NBQX also reduces glutamate mediated brain edema in the rat (Westergren et al., Brain Res. **573**: 324, 1992) and has an anticonvulsant effect in mice (Chapman et al. Epilepsy Res. **9**:92, 1991 and Smith et al., Eur. J. Pharmacol., **201**:179, 1991).

It has now been found that the effective utility of NBQX as an antiischemic agent has been materially improved by administration simultaneously,

separately or sequentially with a diuretic, more specific a loop-diuretic such as furosemide.

Furosemide, 5-(aminosulfonyl)-4-chloro-2-[(2-furanylmethyl)amino]benzoic acid, is described in US 3,058,882.

Furosemide is a highly potent diuretic agent which act on the luminal surface of the acsending loop of Henle by inhibiting the active reabsorbtion of chloride. Along with chloride, there is an enhanced excretion of sodium, potassium, protons, calcium, magnesium, ammonium, bicarbonate and possibly phosphate. The resulting low osmolality in the medulla, decreases the ability of the kidney to reabsorb water. The effect on pH is low, although temporary increases have been observed.

The average peak diuretic response to furosemide in rats and humans are about 30 times the control values of 70 µl/min/kg and 15 µl/min/kg, respectively (Andreasen, 1991).

More specifically, the present invention relates to a method of protection against neuronal cell death associated with neuronal focal or global ischemia, oedema, or traumatic events. Such conditions occur in subjects suffering from stroke, cardiac arrest, suffocation or traumatic injury of the brain or spinal cord. Further, the present invention relates to pharmaceutical compositions useful in the treatment of such conditions.

In particular the invention relates to a method of treating ischemia and related conditions in mammals, including humans, which comprises simultaneous, separate or sequential administration of NBQX and of an effective loop-diuretic, such as furosemide, to a patient suffering from the noted conditions, and to pharmaceutical compositions containing effective doses of both of these compounds.

In addition to furosemide other diuretics, in particular loop-diuretics, which are potent in improving the antiischemic effect of NBQX, are bumetanid, 3-(aminosulfonyl)-5-(butylamino)-4-phenoxybenzoic acid or ethacrynic acid, [2,3-dichloro-4-(2-methylene-1-oxobutyl)phenoxy]acetic acid.

Alternatively, instead of increasing diuresis, another possibility is to increase the solubility of NBQX in urine (NBQX is more soluble at high pH) by diuretics which are carbonic anhydrase inhibitors such as chlorothiazide, hydrochloride or benzthiazide.

This invention is based on the discovery that simultaneous, separate or sequential administration of NBQX and a diuretic agent, such as furosemide, preferably in two separate compositions containing effective amounts of the two compounds, significantly increases the safety margin between the dose causing a potential risk of precipitation of NBQX in the renal tubuli and the dose required for the neuroprotective effect.

The safe dose of NBQX which can be administered is 5-30 times higher than without furosemide, resulting in an increased plasma and brain concentration of NBQX.

Alternatively a loading dose of furosemide followed by the administration of both compounds can be used.

The increased diuresis causes loss of water and salts, in particular sodium and potassium which may be replaced by i.v. infusion of physiological saline or Ringer solution.

The systemic administration and determination of NBQX in rats was performed as follows:

Animal model of ischemia:

Male Wistar rats (average weight 370 g) were fasted overnight with free access to water. Anaesthesia was induced with a mixture of N_2O/O_2 (70%/-30%) containing 3.5% halothane. The animals were intubated and artificially ventilated with the mixture containing 1.2% halothane. Catheters were inserted in the tail artery and in the right internal jugular vein for arterial blood sampling and pressure measurements and for removal/infusion of blood. Arterial blood gases, pH and glucose levels were regularly mesured pre- and post-ischemia. The settings of the ventilator were adjusted to maintain $pACO_2$ between 4.5-5.5 MPa. Rectal temperature was maintained at 37°C by a heat blanket and brain temperature (as monitored by a microsensor positioned in the calvarium below the temporal muscle) was kept at 36.5°C throughout the experimental procedure by a heat lamp.

Induction of ischemia, drug treatment and histological analysis:

Mean arterial pressure (MAP) was reduced to 50 mmHg by removal of blood and subsequently the pneumatic neck tourniquet was inflated to 350 mmHg. Halothane was disconnected at the onset of ischemia. During the ischemic period MAP was maintained at 50 mmHg by infusion/withdrawal of blood. After 10 min, the tourniquet was deflated and the remaining blood rapidly infused. Then rats received either NBQX or physiological saline. The rats were extubated about 60 min. after reperfusion.

Four days after the ischemic challenge, the rats were anaesthetized with pentobarbital and perfusion fixed with 4% buffered paraformaldehyde. After embedding, the brains were coronally sectioned and stained with haematoxylin and eosin. Damage was assessed in a "blind" manner in hippocampus CA1 region, and other regions by counting the number of dead neurones (eosinophilic) as well as normal neurones. Results were compared using two-tailed Student's t-test. (Kaiser et al., Mol. Neuropharm, 2:219-220

1991.

The effect of simultaneous dosing of NBQX and furosemide is demonstrated in the following manner by administration in rats:

Animals

Male Sprague-Dawley rats weighing between 180 - 300 g were anesthetized with an intraperitoneal injection of tribromoethanol in 0.9 % saline (also containing 8 % ethanol) and then cannulated in the right jugular vein. The cannula were transferred subcutaneously, externalised in the neck and connected to a swirvel. The animals were placed in a metabolism cage and allowed to recover for one day before the infusion experiments were started. Dosing was performed as described below and then after 24 hours the cannula was removed and the animals put in an ordinary cage with free acces to food and water for 4 days. Then the rats were killed, the kidneys removed and put in formalin until fixation and histopathologic examination.

Dosing of animals by i.v. infusion

The rats were initially given a solution of 3.8 mg/ml KCl + 6 mg/ml NaCl (at a rate of 25 ml/kg/h) for one hour in order to increase the water load. Then, a solution of 10 mg/ml furosemide was given for 5 min at a rate of 24 ml/kg/h corresponding to a dose of 20 mg/kg. Immediately afterwards was given a formulation containing NBQX (1.0 mg/ml) and furosemide (0.67 mg/ml) in a vehicle consisting of PVP 12 (5 %) and glucose (4%) adjusted to pH 7.4. The rate of infusion was 30 + 60 + 2 ml/kg/h correponding to 30 + 60 + 2 mg/kg/h for ½ + ½ +23 hours, respectively (Fig. 1). In all, a volume of 72 ml/kg was given for the first two hours (one hour pre dosing + the first hour of dosing).

The infusion solutions were introduced through the vein cannula using a gastight glass syringe fitted into a Harvard 22 infusion pump.

Sampling

Urine was collected before (-1 - 0 hour) furosemide was given and then at intervals between 0 - 1, 1 - 3, 3 - 24 hours.

Blood samples were taken from treated animals. After one hour, by the end of the NBQX infusion at highest rate (60 mg/kg/h), the animals were put in light CO_2 anesthesia and blood was collected by heart puncture.

Instrumentation

The HPLC system consisted of a Kontron 420 pump,a Kontron 425 gradient former, a Kontron 460 autosampler, a LiChrospher RP 18, endcapped 5 µm particle analytical column (250 x 4 mm i.d.), a precolumn (4 x 4 mm i.d.) containing the same material. The column was thermostatted in a Kontron 480 oven at 40 °C. Detection was performed with a Kontron 432 UV-detector at 294 nm using Range 0.1 and response time 0.5. A Kontron 450 MT1 data system was used to control the HPLC units and for data aquisition. The mobile phase consisted of tetrahydrofuran:13 mM phosphoric acid adjusted to pH 2.35 (with NaOH) in a 15:85 v/v mixture. The flow rate was 1.0 ml/min.

Extraction procedures

NBQX in plasma:

Bond-Elut C_8 extraction columns (500 mg, 3 ml) were conditioned and eluted using a gentle suction on the outlet. Each column was rinsed twice with 1 ml of methanol and twice with 1 ml of water, followed by 200 µl of 13

mM phosphoric acid (pH 1.85). A 500 µl sample of plasma, 50 µl of internal standard (1 µg/ml) and 500 µl of 8M urea in 13 mM phosphoric acid were added and the columns were submitted to gentle suction. A volume of 1 ml of 13 mM phosphoric acid was then added and suction applied again. Collecting tubes (10 ml glass tubes with conically shaped bottoms) were placed under the extraction columns and 2 ml of methanol was used for eluation. The eluate was evaporated under nitrogen at 60 °C. The residue was redissolved in 100 µl of the mobile phase and centrifuged at 2000 x g for 5 min. The supernatant was transferred to 200 µl vials and capped. A volume of 25 µl of the sample was injected on the HPLC system.

NBQX in urine:

Urine was diluted 1:1 with the mobile phase used for HPLC and 10 µl was injected on the HPLC system using the same chromatographic conditions as used with plasma. Aqueous NBQX solutions were used as standards while controls consisted of urine spiked with NBQX. The results of the NBQX urine concentration, excreted dose, urine volume and pH are summarized in table 1. The urine concentration versus the pH is plotted in fig 2. The concentration is below approx. 200 µg/ml in all fractions including the 0 - 1 hours and the 1 - 3 hours fractions where high concentrations of NBQX could be expected, if not furosemide was administered. Similar concentrations were observed in another study where NBQX was given alone, but at much lower dose rate (1.3 mg/kg/h). In that same study, NBQX related toxicity was not observed after 1 month of dosage.

A large proportion of NBQX is excreted unchanged in urine (56.4 ± 10.9 %).

The plasma concentration of blood samples taken after 1 hour of infusion were similar to those found in the ischemia study which proved NBQX to be neuroprotective. (Fig. 3).

It is concluded that furosemide administered together with NBQX, is likely to protect the kidneys from NBQX related toxicity following doses which are neuroprotective, because furosemide inhibits the reabsorbtion of water in the kidneys, resulting in a high urine flow and consequently a lower concentration of NBQX in urine.

TABLE 1. NNC 07-9202 URINE CONCENTRATION AND EXCRETED DOSE, URINE VOLUME AND PH FROM RATS GIVEN AN I.V. INFUSION OF FUROSEMIDE AND NNC 07-9202 IN A NEUROPROTECTIVE DOSE (30 + 60 +2 MG/KG/H FOR ½ + ½ +23 H)*.

Rat No.	0-1h	1-3h	3-24 h	0-1h	1-3h	3-24 h	0-1h	1-3h	3-24 h	% of Dose
	conc.(µg/ml)			volume (ml)			pH			
1	109.2	162.4	87.4	16.8	3.8	19.6	7.13	6.67	9.01	51.1
2	120	117.8	76.2	17.7	3.6	18.2	7.3	6.84	7.86	47.8
3	103	127	162.4	35.2	9.9	19	7.15	6	6.6	60.2
4	176.4	177.4	41	27.2	6.8	8.3	7.86	7.01	6.83	49.6
5	168	172	222.2	20	4.3	16.8	7.59	5.56	6.31	79.5
6	167.2	157.4	195.6	17.3	4.8	9.3	7.62	6.82	7.2	66.8
7	135.6	166.6	0	19.3	4.5	20.4	8.08	6.84		40.9
8	109	104	189	17.6	6	8.8	7.21	6.25	7.22	50.5
9	121.6	178.4	142.6	19.5	4.1	12.8	7.22	5.95	6.62	60.5
10	63	152	200.6	23.8	4.8	11.4	7	6.79	8.11	50.4
11	108.4	173.8	209.2	21.8	3	13.5	7.75	6.97	6.67	68.6
12	118.2	129.2	69	21	4.5	20.6	7.63	7.1	6.86	51.4

* Urine collected one hour before dosing (i.e. -1 - 0 hours) amounted to 4.3 - 4.6 ml.

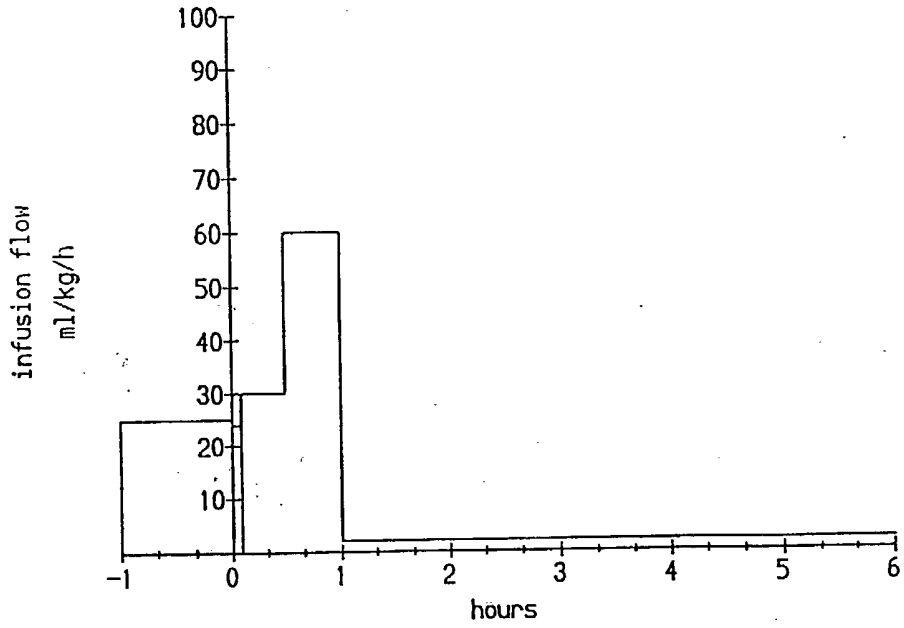

FIG 1. Rats (n = 12) were given a one hour infusion of a physiologic solution of KCl and NaCl before furosemid was administered. Then followed, a 5 min infusion of furosemide (20 mg/kg) and a combination of furosemide (0.67 mg/ml) and NBQX (1 mg/ml). The dose rate of NBQX was 30 + 60 + 2 mg/kg/h for ½ + ½ + 23 hours respectively.

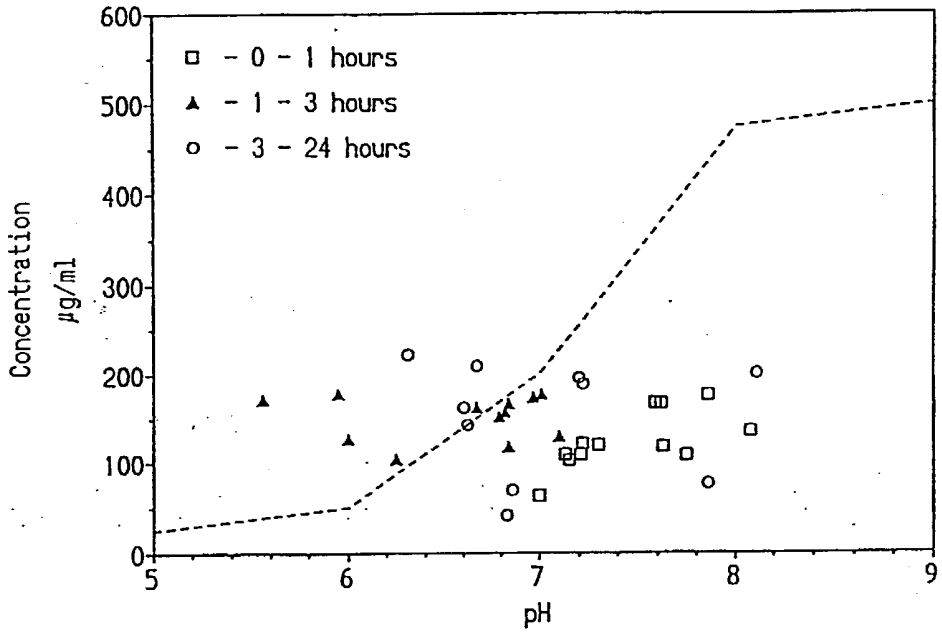

FIG 2. NBQX URINE CONCENTRATION AND pH IN URINE COLLECTED FROM RATS GIVEN FUROSEMIDE AND NBQX BY I.V. INFUSION (30 + 60 + 2 MG/KG/H FOR ½ + ½ + 23 H). THE DOTTED LINE REPRESENT THE SOLUBILITY OF NBQX IN URINE.

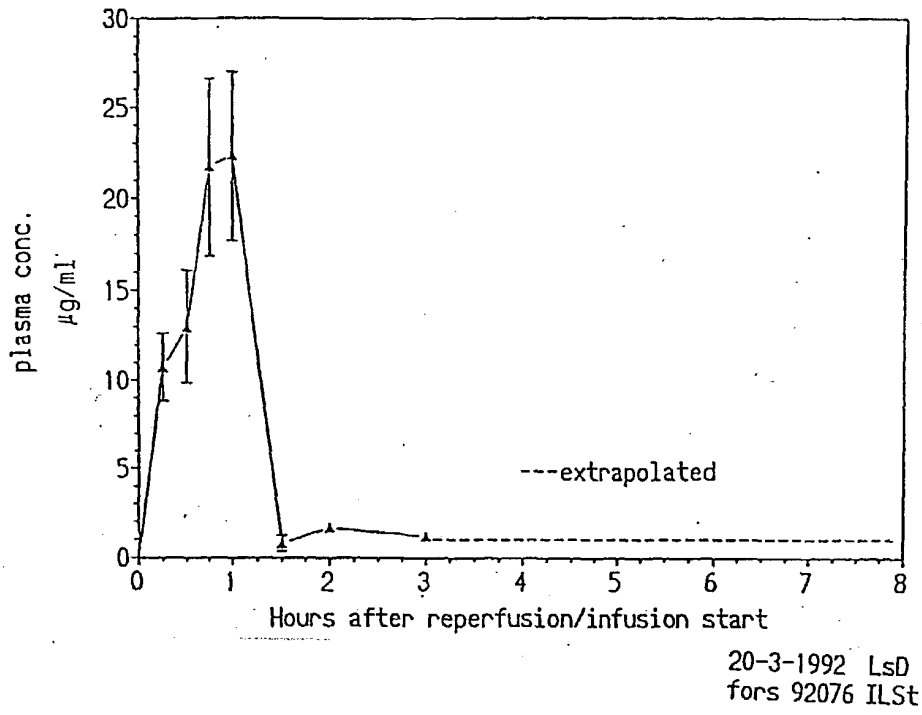

FIG 3. NBQX PLASMA CONCENTRATION - TIME CURVE FROM RATS INCLUDED IN A MODEL OF GLOBAL ISCHEMIA (NECK CUFF STUDY). THE DOSE REGIMEN WAS 30 + 60 +2 MG/KG/H FOR ½ + ½ + 23 H, RESPECTIVELY.

The pharmaceutical preparations or compositions comprising the compounds of the invention may be administered to humans or animals by oral and preferably by intravenous administration. The loop diuretic agent, e.g. furosemide can be given either by the oral or intravenous administration while NBQX is given by intraveneous administration alone.

An effective amount of the active compounds may be determined in accordance with the usual factors, such as the nature and severity of the condition and the weight of the mammal requiring treatment.

Conventional excipients are such pharmaceutically acceptable organic or inorganic carrier substances which do not deleteriously react with the active compounds.

Examples of such carriers are water, salt solutions, alcohols, polyethylene glycols, polyhydroxyethoxylated castor oil, glucose and other carbohybrates, gelatine, lactose, amylose, magnesium stearate, talc, silicic acid, fatty acid monoglycerides and diglycerides, pentaerythritol fatty acid esters, hydroxymethylcellulose, and polyvinylpyrrolidone.

The pharmaceutical preparations can be sterilized and mixed, if desired, with auxiliary agents, such as preservatives, stabilizers, wetting agents, emulsifiers, salt for influencing osmotic pressure, buffers and/or colouring substances and the like, which do not deleteriously react with the active compounds.

Generally, the dosage of the compounds according to this invention is 0.1 mg-4 g/day of NBQX and 0.1 mg-4 g/day of the diuretic agent.

The compounds of the invention, together with a conventional adjuvant, carrier or diluent, may be placed into the form of phamaceutical compositions and unit dosages thereof.

A preferred dosage form for intravenous infusion consists of NBQX (3 μg/ml-3 mg/ml) and furosemide (25 μg/ml-25 mg/ml) and the carriers Polyvidone Ph.Eur. (5%) and glucose (4%) in an aqueous solution made basic with a small excess of sodium hydroxide and then adjusted to pH 7 - 8 with hydrochloric acid. The content of PVP and glucose can be varied in order to obtain an isotonic solution. In order to obtain a quick onset of action of furosemide and consequently of NBQX a loading dose of furosemide alone can be given in the same vehicle as described above or by oral administration using a conventional dosage form.

CLAIMS

1. A pharmaceutical composition suitable for the prevention of neuronal cell death which comprises 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline, a diuretic agent and suitable carriers.

2. A pharmaceutical composition according to claim 1 wherein the diuretic agent is furosemide.

3. A pharmaceutical composition according to claim 1 or 2 wherein the neuronal cell death is associated with focal ischemia.

4. A pharmaceutical composition according to claim 1 or 2 wherein the neuronal cell death is associated with global ischemia.

5. A pharmaceutical composition according to claim 2-4 in the form of an intravenous infusion consisting of 3 μg/ml-3 mg/ml of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline, 25 μg/ml-25 mg/ml furosemide, and the carrier polyvinylpyrrolidone and glucose.

6. A pharmaceutical composition according to claim 2-4 in the form of an intravenous infusion unit containing 0.1 mg-4 g/day of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline, 0.1 mg-4 g/day of furosemide and suitable carriers.

7. A method of preventing neuronal cell death which comprises intraveneous infusion of an effective amount of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline, a diuretic agent and suitable carriers administered optionally together with water, nutrients and salts, preferably sodium, potassium, calcium or magnesium salts.

8. A method of preventing neuronal cell death which comprises intraveneous infusion of an effective amount of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline, an diuretic agent and suitable carriers administered optionally together with water, nutrients and salts, preferably sodium, potassium, calcium and magnesium, characterized in that 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline, the diuretic agent and the salts are administered separately.

9. A method according to claim 7 or 8 wherein the diuretic agent is furosemide.

10. A method according to claim 7, 8 or 9 wherein the neuronal cell death is associated with focal ischemia.

11. A method according to claim 8 or 9 wherein the neuronal cell death is associated with global ischemia.

12. A method according to claim 9-11 wherein the intravenous infusion consists of 3 μg/ml-3 mg/ml of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)quinoxaline, 25 μg/ml-25 mg/ml furosemide, and the carrier polyvinylpyrrolidone and glucose.

13. A method according to claim 9-11 wherein the intraveneous infusion unit contains 0.1 mg-4 g/day of 2,3-dihydroxy-6-nitro-7-sulfamoyl-benzo(f)-quinoxaline, 0.1 mg-4 g/day of furosemide and suitable carriers.

14. A method of preventing neuronal cell death which comprises an oral loading dose of furosemide and subsequently intraveneous infusion of an effective amount of 2,3-dihydroxy-6-nitro-7-sulfamoylbenzo(f)quinoxaline and furosemide.

15. A method according to claim 14 wherein the loading dose is given in the form of an intraveneous administration.

16. A method of preventing neuronal cell death in a subject in need thereof comprising administering to said subject a pharmaceutical composition according to claim 1 or 2.

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00101

A. CLASSIFICATION OF SUBJECT MATTER

IPC5: A61K 31/495, A61K 31/34
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC5: A61K

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

SE,DK,FI,NO classes as above

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

CA, MEDLINE, EMBASE, US PATENT FULLTEXT

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	MOLECULAR PHARMACOLOGY, Volume 41, 1992, Juan Lerma et al, "Chloride Transport Blockers Prevent N-Metyl-D-aspartate Receptor-Channel Complex Activation" page 217 - page 222 --	1-6
A	European Journal of Pharmacology - Molecular Pharmacology Section, Volume 244, 1993, Aöndrew J. Palmer et al, "Cyclothiazide reverses AMPA receptor antagonism of the 2,3-benzodiazepine, GYKI 53655" page 193 - page 194 -- ---------	1-6

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
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"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance: the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance: the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
8 July 1994	14 -07- 1994
Name and mailing address of the ISA/ Swedish Patent Office Box 5055, S-102 42 STOCKHOLM Facsimile No. +46 8 666 02 86	Authorized officer Solveig Gustavsson Telephone No. +46 8 782 25 00

Form PCT/ISA/210 (second sheet) (July 1992)

INTERNATIONAL SEARCH REPORT

International application No.
PCT/DK 94/00101

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. [X] Claims Nos.: 7-16
because they relate to subject matter not required to be searched by this Authority, namely:

See PCT Rule 39.1(iv): Methods for treatment of the human or animal body by surgery or therapy, as well as diagnostic methods.

2. [ ] Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. [ ] Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. [ ] As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. [ ] As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. [ ] As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. [ ] No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

[ ] The additional search fees were accompanied by the applicant's protest.

[ ] No protest accompanied the payment of additional search fees.